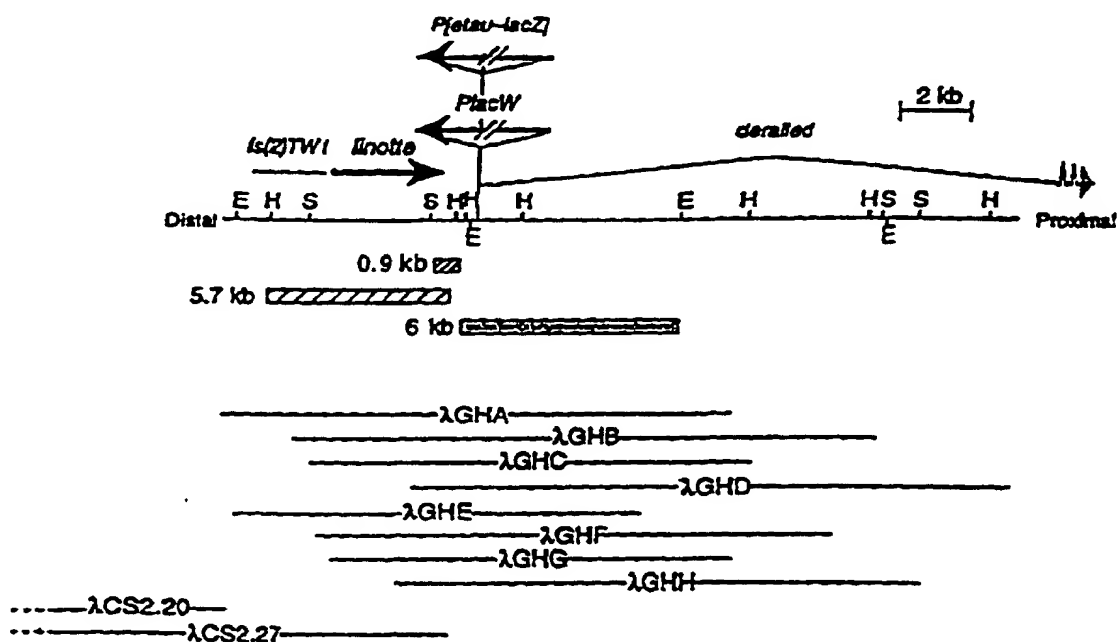




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(54) Title: ASSOCIATIVE LEARNING AND THE LINOTTE GENE



(57) Abstract

The invention pertains to a novel gene present in cytological region 37D of the second chromosome which functions in associative learning and/or memory. Disruption of the gene, such as by P element transposon tagged insertion, results in decreased associative learning and/or memory. The invention also pertains to a novel protein encoded by the gene, antibodies which bind the encoded protein, and homologs of the novel gene which function in associative learning and hybridize to the DNA sequence of the novel gene.

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ASSOCIATIVE LEARNING AND THE *linotte* GENEBackground of the Invention

Behavioral analyses of associative learning and memory have revealed a general functional homology among
5 invertebrates and vertebrates (reviewed by Hawkins and Kandel, *Psychol. Rev.* 91:375-391 (1984); DeZazzo and Tully, *Trends Neurosci* 18:212-217 (1995); and Hammer and Menzel, *J. Neurosci* 15:1617-1630 (1995)). Acquisition requires the temporal association of a reinforcing
10 stimulus (US), which naturally elicits a behavioral response, with a conditioned stimulus (CS), which comes to elicit a conditioned response (CR) as a result of the CS-US pairing(s) (Mackintosh, *Conditioning and Associative Learning* (New York: Oxford University Press)
15 (1983)).

A newly acquired experience initially is susceptible to various forms of disruption. With time, however, the new experience becomes resistant to disruption (McGaugh and Herz, *Memory Consolidation* (San
20 Francisco: CA: Albion) (1972); Tully et al., *Cold Spring Harbor Symp. Quant. Biol.* 55:203-211 (1990)). This observation has been interpreted to indicate that a labile, short-term memory is "consolidated" into a more stable, long-term memory. This consolidation process
25 depends on protein synthesis (Davis and Squire, *Psych. Bull.* 96:518-559 (1984); Castellucci et al., *J. Neurobiol.* 20:1-9 (1989); Crow and Forrester, *Proc. Natl. Acad. Sci. USA* 87:4490-4494 (1990); Tully et al., *Cell* 79: 35-47 (1994)) and is facilitated by multiple
30 training sessions separated by intervals of rest (Carew et al., *Science* 175:451-454 (1972); Frost et al., *Proc.*

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Natl. Acad. Sci. USA 82:8266-8269 (1985); Huang and Kandel, *Learn. Mem.* 1:74-82 (1994); Tully et al., *Cell* 79:35-47 (1994)).

This behavioral homology appears to reflect in part
5 an underlying molecular homology. Genetic dissection of
olfactory associative learning in fruit flies (reviewed
by Davis, *Neuron* 11:1-14 (1993); DeZazzo and Tully,
Trends Neurosci. 18:212-217 (1995)) and cellular
analyses of heterosynaptic facilitation in *Aplysia*
10 (reviewed by Byrne et al., *In Advances in Second
Messenger and Phosphoprotein Research* S. Shenolikar and
A.C. Nairn, eds. (New York: Raven Press) pp. 47-107
(1993)) or synaptic long-term potentiation in
vertebrates (reviewed by Bliss and Collinridge, *Nature*
15 361:31-39 (1993); Eichenbaum and Otto, *Neurosci.* 16:22-
24 (1993)) all have revealed the involvement of the cAMP
second messenger system. Temporally paired stimuli
induce an increase in cAMP (Wu et al., *Proc. Natl. Acad.
Sci. USA* 92:220-224 (1995)) which activates a cAMP-
20 dependent protein kinase (PKA). PKA then serves two
functions. In the cytoplasm, activated PKA
phosphorylates targets, such as ion channels, thereby
modulating synaptic efficacy for minutes to hours (Cowan
and Siegel, *J. Neurogenet.* 3:187-201 (1986); Skoulakis
25 et al., *Neuron* 11:197-208 (1993)). Activated PKA also
is translocated to the nucleus, where it phosphorylates
a cAMP responsive transcription factor, CREB (Dash et
al., *Nature* 345:718-721 (1990); Yin et al., *Mol. Cell.
Biol.* in press (1995)). Phosphorylated CREB (activator)
30 then initiates a cascade of immediate early genes,
including C/EBP (Alberini et al., *Cell* 76:1099-1114
(1994); Bourtschuladze et al., *Cell* 79:59-68 (1994); Yin
et al., *Cell* 79:49-58 (1995)), presumably culminating in
a protein synthesis-dependent synaptic growth process
35 (Greenough, *Neurosci.* 7:229-283 (1984); Stewart and
Rusakov, *Behav. Brain Res.* 66:21-28 (1995)). This
physical change at the synapse may be responsible (at

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least in part) for more long-lasting modulations of synaptic efficacy and long-term memory.

All the above observations suggest an evolutionarily conserved molecular mechanism involved with the formation of long-term memory: learning-induced activation of the cAMP second messenger system, which terminates in a CREB-mediated transcription factor cascade involved with synaptic growth and function. Although this process may represent a core mechanism common among many species, many other molecules appear to be involved, especially with short-term plasticity (Malinow et al., *Science* 245:862-866 (1989); Abeliovich et al., *Cell* 75:1263-1271 (1993); Mihalek et al., (submitted) (1995)). These observations suggest that the cAMP pathway may be involved only in certain learning tasks and/or that it is more generally necessary but perhaps not sufficient. Indeed, flies homozygous for null mutations of the *dunce* or *rutabaga* genes, which encode a cAMP-specific phosphodiesterase and a calcium/calmodulin-dependent adenylyl cyclase, respectively, nevertheless display significant residual associative learning (Tully and Quinn, *J. Comp. Physiol. A.* 157:263-277 (1985); Tully and Gold, *J. Neurogenet.* 9:55-71 (1993)). Thus, the molecular and behavioral intricacies of learning and memory suggest that additional genes remain to be discovered which may participate in these processes.

Summary of the Invention

About 2200 P element insertional (transposon-tagged) *Drosophila* lines were generated and screened for reduced 3 hour memory retention after Pavlovian olfactory learning. The behavior-genetic characterization of two new genes, *latheo* and *linotte*, identified from this screen have been described (Boynton and Tully, *Genetics* 131:655-672 (1992); Dura et al., *J. Neurogenet.* 9:1-14 (1993)). Mutant *latheo* and *linotte*

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flies are affected in acquisition of conditioned odor avoidance responses and in rather than memory retention thereafter. Moreover, transposon-tagging these genes allowed their expeditious molecular cloning.

5 As described herein, the molecular identification of the *linotte* transcription unit has been achieved. The *linotte* transcription unit was identified via rescue of the *lio*¹ learning/memory defect by induced expression of a *lio*¹ transgene in adults. The perception of odors
10 or electroshock remained normal when the *lio*¹ transgene was expressed in these *lio*¹ flies. Learning/memory remained normal when the *lio*¹ transgene was expressed in wild-type (*lio*⁺) flies.

Only one message is detected throughout the
15 development of wild-type flies, and the level of this transcript is reduced in adult *linotte* mutants. Sequence analysis of a cDNA clone (SEQ ID NO: 1) corresponding to this mRNA has revealed one 2.7 kb *lio*¹ open reading frame (ORF). Heat-induced expression of a
20 *hsllo*¹ transgene three hours before training fully and specifically rescues the learning and memory defects of *linotte* mutants. These data constitute definitive proof that the correct (*linotte*) transcription unit has been identified. The deduced amino acid sequence (SEQ ID NO:
25 2) of this transcript bears no homology to any known protein, indicating that the *linotte* gene encodes a novel protein involved with associative learning.

In one embodiment of the invention, the novel gene comprises the nucleotide sequence of SEQ ID NO: 1 (the
30 *linotte* or *lio* gene). The learning/memory defect caused by disruption of the *linotte* gene can be specifically and completely rescued by expression of a heat shock promoter *lio*¹ transgene.

The invention also pertains to a protein encoded by
35 a gene of the present invention. In one embodiment, the protein has the amino acid sequence of SEQ ID NO: 2.

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The invention also pertains to antibodies which bind to the protein encoded by a gene of the present invention.

The invention further pertains to a gene present in cytological region 37D of the second chromosome which
5 functions in associative learning and/or memory and which hybridizes under standard conditions to the DNA sequence of SEQ ID NO: 1 or the complement of SEQ ID NO:1.

The present invention further provides methods for
10 identifying other genes which function in learning and memory from a variety of organisms, including vertebrates (e.g., mammals and particularly humans), invertebrates (e.g., insects) and microbes (e.g., yeast). Furthermore, comparison of *linotte* and other
15 learning and/or memory genes and their encoded products provides a way to define key functional features or regions of these genes and gene products. Those features or parts that are conserved between these genes or their gene products are most likely to be
20 functionally important.

The present invention has several applications pertaining to learning and memory. The genetic and molecular characterization of *linotte* herein can be used to design drugs which mimic or alter the activity of the
25 *linotte* or other learning and memory genes, and which may, thus, be useful in various therapeutic applications, including learning disabilities, memory defects associated with Alzheimer's disease and other afflictions which are associated with decreased learning
30 and memory.

The complete or partial DNA or amino acid sequence of the *linotte* gene can also be used as a probe to identify other genes which function in learning and/or memory. The present invention provides methods for
35 identifying other genes which function in learning and memory from a variety of organisms, including vertebrates (e.g., mammals and particularly humans),

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invertebrates (e.g., insects) and microbes (e.g., yeast).

Brief Description of the Drawings

Figure 1 depicts a schematic map of genomic DNA in cytological region 37D of the second chromosome, which contains the *linotte* gene. The restriction fragment map shows sites for *EcoRI* (E), *HindIII* (H) and *Sac II* (S). The *PlacW* P element transposon, which causes the *lio¹* mutation, is depicted as a grey arrow indicating the transcriptional orientation of the *lacZ* reporter gene; its point of insertion in the genome is marked by a descending line. The *linotte* transcript is indicated by a black arrow. The *P[etau-lacZ]* P element, which causes the *derailed* mutation, is represented by a grey arrow indicating the transcriptional orientation of the *etau-lacZ* reporter gene. The 5.7 kb *HindIII*-*HindIII*, the 6 kb *EcoRI*-*EcoRI* and the 0.9 kb *SacII*-*HindIII* genomic restriction fragments, which were used to probe cDNA or genomic libraries, are represented by boxes below the restriction map. Genomic phage clones (λ) identified either with the 0.9 kb *SacII*-*HindIII* restriction fragment (λ GHA- λ GHH) or from the *Ddc* project (λ CS2.20 and λ CS2.27) are indicated as lines below.

Figure 2 illustrates the 3098 nucleotide complementary DNA sequence of the *linotte* gene (SEQ ID NO: 1).

Figures 3A and 3B are graphic representations of the rescue of learning and memory deficits of *linotte¹* (*lio¹*) mutants by heat shock-induced expression of the *hslio⁺* transgene. Figure 3A shows conditioned odor avoidance immediately after olfactory learning in wild-type (Can-S), mutant *lio¹* (*lio*) and transgenic *lio¹;hslio⁺-16* (*lio;16*) and *lio¹;hslio⁺-3* (*lio;3*) flies in the absence of heat-shock (-hs) or three hours after a 30-minute heat-shock (+hs). Figure 3B shows memory retention three hours after olfactory learning (three

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hour retention) in wild-type (Can-S), mutant *lio*¹ (*lio*) and transgenic *lio*¹;*hsl**lio*⁺-16 (*lio*;*16*) and *lio*¹;*hsl**lio*⁺-3 (*lio*;*3*) flies trained in the absence of heat-shock (-hs) or three hours after a 30-minute heat-shock (+hs).

5 Figures 4A and 4B are graphic representations of the effect on learning and memory in *lio*⁺ (wild-type) flies of heat shock-induced expression of the *hsl**lio*⁺ transgene. Figure 4A shows conditioned odor avoidance immediately after olfactory learning in wild-type (Can-S) and transgenic *lio*⁺;*hsl**lio*⁺-16 (*lio*⁺;*16*) flies in the
10 absence of heat-shock (-hs) or three hours after a 30-minute heat-shock (+hs). Figure 4B shows memory retention three hours after olfactory learning in wild-type (Can-S) and transgenic *lio*⁺;*hsl**lio*⁺-16 (*lio*⁺;*16*)
15 flies trained in the absence of heat-shock (-hs) or three hours after a 30-minute heat-shock (+hs).

 Figures 5A and 5B show the open reading frame (ORF) and deduced amino acid sequence of the *linotte* gene. Figure 5A depicts ORF maps of each reading frame of the
20 *lio*⁺ sense strand. All ORFs initiating with an AUG and longer than 100 nucleotides (nt) are indicated with arrows above a linear representation of the *lio*⁺ cDNA. Figure 5B depicts the deduced 916 amino acid sequence (SEQ ID NO: 2) of the 2748 nt *lio*⁺ ORF.

25 Figure 6 shows a graphic summary of planimetric determinations of mean (\pm SEM) neuropilar volumes of the mushroom body calyces and central complexes of wild-type (+/+), +/*Df*, *lio*¹/*Df* and *lio*¹/*lio*¹ males and females.

Detailed Description of the Invention

30 The invention described herein pertains to a novel gene, comprising the DNA sequence of SEQ ID NO: 1, present in cytological region 37D of the second chromosome and which functions in associative learning and/or memory. Disruption of the gene, such as by P
35 element transposon-tagged insertion, results in decreased associative learning and/or memory.

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The teachings of references cited herein are incorporated herein by reference in their entirety. Terms used herein are intended to have their art-recognized meanings unless otherwise indicated. As used

5 herein, the term "disruption" of a gene is intended to mean any alteration of the nucleotide sequence of the gene or the amino acid sequence of the encoded protein from the wild type, including a frameshift, insertion or deletion.

10 Two transgenic lines (*lio*⁺*;hsl**io*⁻-16 and *lio*⁺*;hsl**io*⁻-3), carrying independently isolated genomic insertions of a heat shock promoter-linotte (*hsl**io*⁺ transgene, showed normal olfactory learning and memory after expression of the transgene was induced by heat shock in

15 adults (Figures 4A and 4B). This rescue effect is behaviorally specific, since induced expression of the *hsl**io*⁺ transgene did not affect the flies' task-relevant abilities to sense the odors (olfactory acuity) or electroshock (shock reactivity) used in the Pavlovian

20 experiments (Table 1; cf. Dura et al., *J. Neurogenet* 9:1-14 (1993)).

It should be noted that any appropriate promoter can be used to drive the expression of the *hsl**io*⁺ transgene; the appropriateness of the promoter will

25 depend on the environment and specifically desired expression. For instance, in some cases it will be desirable to create a *lio*⁺ transgene driven by an inducible promoter such as the heat shock or metallothionein promoters. In other situations it will

30 be desirable to drive expression of the *lio* transcript using a constitutive promoter. For instance, when expressing the Lio protein in cell culture, the desired promoter can vary depending upon the purpose of the protein expression.

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Complete rescue of the *lio*¹ learning/memory defect in induced *hsllo*⁺ flies indicated that ectopic expression of the *lio*⁺ transgene does not produce any deleterious effects on conditioned olfactory behavior. The other extreme also was considered; that is, that (ectopic) expression of the *hsllo*⁺ transgene might produce a general enhancement, thereby improving learning/memory in *lio*¹ mutants nonspecifically. This possibility was tested by inducing overexpression of the *hsllo*⁺ transgene in *lio*⁺ (wild-type) flies, rather than in *lio*¹ mutants. In such transgenic *lio*⁺;*hsllo*⁺-16 flies, learning/memory was normal. Thus, induced expression of the *hsllo*⁺ transgene did not produce a general enhancement of learning or memory. Consequently, it can be concluded that the deleterious effects on learning and memory of the *lio*¹ mutation specifically were rescued by induction of the *hsllo*⁺ transgene.

In the absence of heat shock, transgenic *lio*⁺;*hsllo*⁺ flies show learning/memory deficits similar to those of *lio*¹ mutants (Figures 4A and 4B), and expression of the transgene is not detected in adult heads. Conditioned odor avoidance after olfactory learning was quantified as a mean Performance Index (PI) $241 \pm \text{SEM}$. If no flies learn to avoid the shock-paired odor, then PI=0; if all flies learn, then PI=100. N=6 PIs per group.

In contrast, three hours after heat shock, learning and memory are rescued completely, and expression of the transgene in adult heads is high. Combined with data from the developmental Northern blot, which revealed undetectable levels of the *linotte* transcript throughout larval development, and from histological studies of mutant adult brain, which revealed no structural abnormalities in mushroom bodies or central complex, these results indicate clearly that the learning/memory deficit of *lio*¹ mutants does not derive secondarily from developmental abnormalities. Instead, the *linotte* gene appears to function more acutely during adult

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associative learning (cf. Ewer et al., Nature 333:82-84 (1988)). This inducible, complete and behaviorally-and molecularly-specific rescue of the *linotte* learning/memory deficit constitutes definitive proof
5 that the *linotte* transcription unit was correctly cloned and identified.

A high level of *lio'* expression in early embryos followed by much lower levels in late embryos suggests that *lio'* initially is maternally derived. Appreciable
10 levels of expression are not observed again until the pupal and adult stages. PolyA+ RNA was extracted from wild-type (Canton-S) early embryos (0-4 hour embryo), late embryos (> 16 hour embryo), first, second or third instar larvae, pupa, adult head and adult body,
15 electrophoresed on a 1% denaturing agarose gel, transferred to a charged nylon membrane and probed with ³²P-radiolabeled *lio'* (3.1 kb) cDNA (L) and *rp49* (R) as described above. When comparing the temporal patterns of expression between the *linotte* transcript and the
20 enhancer-trap reporter gene encoded within the *lio'* P element insertion, an apparent discrepancy exists. The former cannot be detected in any larval stage, while the latter is expressed at high levels in the lateral brain hemispheres of third-instar larvae (data not shown).
25 Recent identification of the *derailed* (*drl*) gene, however, has revealed a more specific resolution to this discrepancy for *lio'* (Callahan et al., Nature (in press) (1995)).

DNA sequence comparison has shown that the
30 identified *lio'* P element is inserted six base pairs distal to the independently isolated P element insertion in *drl* (Figure 1). The *derailed* transcript lies proximal (right), and the *fs(2)TW1* transcript lies distal (left), to the *linotte* transcript. Consistent
35 with this finding, the embryonic CNS patterns of reporter gene expression for both P element insertions correspond to the expression pattern of the *drl* gene

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itself (C. Cahallan and J. Thomas, personal comm.). Moreover, the *derailed* transcript is expressed throughout larval development but is undetectable in adult flies. The latter result is consistent with our
5 Northern blot analysis of adult head RNA, in which a genomic DNA fragment proximal to the *lio*¹ P element insertion (and including at least some of the *derailed* exonic sequence) failed to detect any transcript.

These data indicate that the *lio*¹ P element affects
10 the level of expression of the *linotte* transcript in adult flies - thereby producing a learning/memory deficit - even though it is inserted in or near the 5' end of the *drl* transcription unit. It is not yet known whether the *lio*¹ P element insertion also produces the
15 *derailed* mutant phenotype, axonal misguidance in a subset of neurons during development of the nervous system in embryos (Callahan et al., *Nature* (in press) (1995)). Nevertheless, these potential pleiotropic defects do not prevent complete rescue of the *lio*¹
20 learning/memory defect after the *hsllo*¹ transgene is induced in adults.

To date, RNA in situ hybridization studies using *linotte* RNA probes have failed to produce any detectable signal in adult brain sections. Immunocytochemical
25 studies using antibodies raised against the *linotte* gene product, however, may reveal its cellular localization.

The *linotte* gene originally was isolated in a behavioral screen for P element insertional mutations that disrupted 3-hour retention after Pavlovian
30 olfactory learning. To date, behavioral characterization of 2200 hundred P element lines has yielded four new learning/memory genes, *linotte*¹, *latheo*^{P1}, *nalyot* and *golovan* (Boynton and Tully, *Genetics* 131:655-672 (1992); Dura et al., *J. Neurogenet* 9:1-14
35 (1993) and T. Tully unpublished data). The transposon-tagging method was chosen for two reasons particularly relevant to behavioral phenotypes. First, the P element

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mutator contained a selectable eye-color marker, which yielded morphologically tagged behavioral mutants. Consequently, subsequent genetic experiments were greatly facilitated. Second, the P element mutator represented a molecular tag with which to clone genomic DNA flanking the insertion site. This approach gained access to genomic DNA in the region of the P element insertion but was not designed to identify unambiguously the transcription unit specifically responsible for the learning/memory deficit.

The particular set of molecular-genetic, histological and behavioral data derived from *linotte* cloning has yielded important information. Insertion of the P element into a transcription unit is not sufficient evidence to conclude that the particular transcript is involved with the behavioral defect. Correspondence between patterns of expression of an enhancer-trap reporter gene (contained within the transposon) and of a nearby transcript also does not constitute sufficient evidence to conclude that the particular transcript is involved with the behavioral phenotype. The only evidence sufficient to draw such a conclusion is rescue of the mutant phenotype by expression of a (wild-type) transgenic transcript, along with controls for behavioral and molecular specificity. Although the issue of behavioral specificity seems trivial in light of the complete rescue of mutant *lio*¹ learning/memory, it becomes quite relevant when only partial rescue of a learning/memory defect is observed, as recently has been reported for the *dunce* gene (Dauwalder and Davis, *J. Neurosci.* 15:3490-3499 (1995)).

This singular criterion is particularly relevant for P element-derived mutations, since these large foreign pieces of DNA are likely to disrupt the expression of several nearby genes. Phenotypic rescue in transgenic flies is sufficient, as well, for ethylmethane sulfonate (EMS)-induced mutations, which

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tend to produce more restricted (even single-nucleotide) molecular lesions. When multiple, independently derived mutant alleles are available, however, a second approach can be used to identify the relevant transcription unit; 5 molecular lesions corresponding to each of several mutations can be shown to reside within the same transcription unit (e.g., *bithorax*; Bender et al., *Science* 221:23-29 (1983)).

Of particular interest is the observation that the 10 *linotte* gene encodes a previously unknown protein. Future studies will reveal the biological function(s) of this new gene. Given the frequency with which learning/memory mutants were identified from our screen (1 mutant per 550 P element lines) and the speed with 15 which the correct transcript was identified for *linotte*, this approach in *Drosophila* appears particularly expeditious. With substantial molecular and behavioral homology for associative learning and memory processes already documented among fruit flies, bees, mollusks and 20 vertebrates (see DeZazzo and Tully, *Trends Neurosci.* 18:212-217 (1995) for a review), trans-species homologs of the *linotte* gene are anticipated.

This evidence suggests that there may be a human equivalent of the *linotte* gene; using *linotte*, other 25 associative learning/memory genes from a variety of organisms can be obtained, for example by hybridization procedures known in the art. The sequences of these related genes and their encoded products can be compared, for instance, using computer-based analysis, 30 to determine their similarities. Structural comparisons, for example, would indicate those regions or features of the genes or encoded products which are likely to be functionally similar and important. Such information can be used to design drugs which mimic or 35 alter the activity of the *linotte* or other learning and memory genes, and which may, thus, be useful in the various therapeutic applications, including learning

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disabilities, memory defects associated with Alzheimer's disease and other afflictions which are associated with decreased learning and memory.

In addition, comparison of equivalent genes and their encoded products, as well as mutational analysis, is expected to indicate key functional features or regions of the genes or gene products. The learning and memory genes and their gene products are further useful for developing and identifying agents which affect the activity of the described associative learning genes. These agents may be useful for altering (increasing or decreasing) the occurrence of learning and/or memory defects in an organism, and thus, altering the learning ability and/or memory capacity of the organism.

The invention will be further illustrated by the following non-limiting examples:

Examples

Plasmid Rescue and cDNA Cloning

Genomic sequences flanking the *linotte* P-element were cloned by plasmid rescue using standard techniques (Sambrook et al., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1989); Wilson et al., *Genes Dev.* 3:1301-1313 (1989)). Briefly, *linotte* genomic DNA was digested with *SacI*, followed by ligation to form a rescue plasmid, which was propagated in *E. coli* LE392. The rescue fragment then was ³²P-radiolabeled by random priming and used to screen 3 x 10⁷ phage plaques from a *Drosophila* genomic bacteriophage λ -DASH library (Sambrook et al., *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1989)). The 5.7 kb *Hind III* λ genomic fragment (Figure 1) was subcloned into the plasmid vector pBS-KS+, radiolabeled and used to probe a Northern blot of adult whole fly polyA+RNA and a λ gt11 *Drosophila* adult head cDNA library. A 6 kb *EcoRI* fragment (Figure 1)

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corresponding to the sequences distal to the *lio*¹ P-element was subcloned into the plasmid vector pBS-KS+, radiolabeled and used to probe a Northern blot of adult head polyA+RNA. A 3.1 kb *EcoRI* *lio* cDNA fragment was excised from the λ page and subcloned into the plasmid vector pBS-KS+. This 3.1 kb insert (containing the 2.7 kb ORF) was cloned into the *EcoRI* (polylinker) site of the transformation vector CaSpeR-has, which contains a white⁺ minigene as a selectable marker and P-element sequences to facilitate insertion into the genomic DNA (Pirrotta, *Biotechn.* 10:437-456 (1988)). This transgene construct was designated *hslio*⁺.

Fly Stocks

The genetic background of *w;lio*¹ mutants was equilibrated with that of the wild-type (Canton-S) strain by repeatedly backcrossing heterozygous *w/w;lio*¹/+ females (which carried the *mw*⁺ eye-color marker) to *w*(CS10) males for more than five generations. The *w*(CS10) strain was derived by backcrossing *w*¹¹⁸ flies to wild-type (Canton-S) flies for 10 generations (Dura et al., *J. Neurogenet.* 9:1-14 (1993)); the *w*(*isoCJ1*) strain was derived from *w*(CS10) and carries isogenic X, 2nd and 3rd chromosomes (Yin et al., *Cell* 79:49-58 (1994); Yin et al., *Cell* 81:107-115 (1995a)).

Homozygous *w;lio*¹ flies (hereafter referred to as *lio*¹) were bred a few months before behavioral experiments. For histological experiments, *lio*¹ homozygotes or another "wild-type" *PlacW* transposant strain, *E₄(TP)* which showed normal olfactory acuity, shock reactivity and olfactory learning, were coded and then crossed to flies carrying the second chromosome deletion *Df(2L)VA12* (hereafter referred to as *Df*) and the *CyO* second chromosome balancer. Straight-winged flies from each cross, *lio*¹ mutants and *E₄(TP)* flies then were processed together and decoded after the planimetric analysis (see below). To generate transgenic flies, approximately

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3000 *w(isoCJ1)* embryos were dechorionated with 40% aqueous bleach for 60 seconds, rinsed in water, desiccated at 18°C and 60% R.H. for approximately 20 minutes, aligned on acetate-based double-sided tape (3M type 415, 3M, St. Paul, MN) and coinjected with *hslio*⁺ and with the transposase-source plasmid pUCHspD2-3wc (Rubin and Spradling, *Science* 218:348-353 (1982); Spradling and Rubin, *Science* 218:341-347 (1982)). Approximately 200 G₀ flies were recovered and mated to *w(isoCJ1)*, from which 18 independent, fertile transformant lines were established. These transgenic lines were designated *lio*⁺;*hslio*⁺-X. Flies from four transgenic lines, carrying *hslio*⁺ insertions in the third chromosome (*lio*⁺;*hslio*⁺-16,*lio*⁺;*hslio*⁺-3,*lio*⁺;*hslio*⁺-21 and *lio*⁺;*hslio*⁺-1), were crossed with *lio*¹ mutants to recover heterozygous *lio*¹/+; *hslio*⁺/+ progeny, which were identified by eye color. These heterozygotes then were mated to a "cantonized" *CyO/Sp;TM6B/Sb* double balancer strain to yield *lio*¹/+; *hslio*⁺/progeny, which were identified by eye color. These heterozygotes then were mated to a "cantonized" *CyO/Sp;TM6B/Sb* double balancer strain to yield *lio*¹/*CyO;hslio*⁺/*TM6B* progeny. Intermating of these flies yielded four lines homozygous for the *lio*¹ mutation on the second chromosome and for the *hslio*⁺ transgene on the third chromosome. These transgenic lines were designated *lio*¹;*hslio*⁺-X.

RNA Isolation and Northern Blotting

Flies were collected and sacrificed immediately by flash-freezing in liquid nitrogen. Where applicable, *Drosophila* adult head and body mRNA was made by vigorously shaking frozen flies and separating the frozen heads and bodies by sifting over dry ice. The frozen parts were pulverized in a mortar on dry ice and then extracted using the acidic guanidinium isothiocyanate method (Chomczynski and Sacchi, *Analytical Biochemistry* 162:156-159 (1987)). PolyA+RNA

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subsequently was selected by oligo(dT)⁺ chromatography (Chirgwin et al., *Biochemistry* 18:5294 (1979)). The polyA⁺RNA then was electrophoresed on a 1% formaldehyde agarose gel, transferred to a charged nylon membrane and

5 probed with the radiolabeled 3.1 kb cDNA fragment or with a fragment from the *Drosophila* ribosomal protein gene 49 (*rp49*) (Church and Gilbert, *Proc. Natl. Acad. Sci. USA* 81:1991 (1984); O'Connell and Rosbash, *Nucl. Acids Res.* 12:5495-5513 (1984)). Two Northern blots

10 were generated, each containing polyA⁺RNA from wild-type flies and *lio*¹ mutants. Bands intensities were determined by phosphorimage analysis (Fuji Photo Film Co.). Intensity of the *lio*⁺ transcript was normalized to that of *rp49* within a given lane (wild-type or mutant).

15 Levels of expression of the *lio*⁺ transcript in *lio*¹ mutants then were expressed as percentages of the levels of expression of the *lio*⁺ transcripts in wild-type flies in each corresponding Northern blot. Finally, these two percentages of expression were averaged.

20 Expression of the *linotte* (*lio*⁺) transcript is reduced in *lio*¹ mutants and is not affected by heat shock, while expression of the *hsllo*⁺ transgene is induced by heat shock (data not shown). In the absence of heat shock, twice as much *lio*⁺ transcript is expressed

25 in wild-type (Canton-S) adult heads as in mutant *lio*¹ heads or in transgenic *lio*¹;*hsllo*⁺-16 heads, while expression of the *hsllo*⁺ transgene was not detected. Three hours after heat shock, when flies were subjected to olfactory learning, expression of the *lio*⁺ transcript

30 was unchanged in wild-type, mutant *lio*¹ or transgenic *lio*¹;*hsllo*⁺-16 flies. In contrast, the *hsllo*⁺ transgene was expressed at a high level. Six hours after heat-shock, when 3-hour memory after olfactory learning was assayed, expression of the *hsllo*⁺ transgene was greatly

35 diminished. PolyA⁺RNA was extracted from wild-type (Can-S), *lio*¹ (*lio*) and *lio*¹;*hsllo*⁺-16 (*lio*;16) adult heads, electrophoresed on a 1% denaturing agarose gel,

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transferred to a charged nylon membrane and probed with ^{32}P -radiolabeled *lio*⁺ (3.1kb) cDNA. To quantify relative amounts of RNA loaded into each lane, this Northern blot was reprobbed with a ^{32}P -radiolabeled DNA fragment from the ribosomal protein 49 (*rp49*) gene. Radiolabeled signals finally were quantified with a phosphorimager, and signal intensities of bands hybridizing with the *lio*⁺ probe were normalized using *rp49* signal intensities from each corresponding lane.

10 Histology

To quantify the neuropilar volumes of mushroom bodies and central complex, paraffin sections of *lio*⁺, *lio*⁺[*E₇4(TP)*], *lio*⁺/*Df* and *lio*⁺/*Df* flies were prepared as by Ashburner (*Drosophila - A Laboratory Manual* (Plainview NY: Cold Spring Harbor Laboratory Pres, 1989) with a few modifications: Heads were first incubated in 1:1 (methylbenzoate:low melting paraffin), followed by six 30 minute incubations with pure paraffin. Seven μm frontal sections were inspected visually at 400X magnification. The volumes of mushroom body or central complex neuropil then were quantified via planimetric analysis using an MTI CCD 725 camera connected to a Screen Machine Classic Videoboard (FAST electronic GbhH, Munich, Germany) in an MS-DOS PC with custom software developed by R. Wolf and M. Heisenberg (Heisenberg 1995). The operator traced the outlines of mushroom body calyces and the central complex (including the noduli and fan-shaped body) through serial sections while blind to genotype.

30 Xgal Staining of Tissues

Embryos were dechorionated, fixed in 3.7% formaldehyde in PBS and X-gal stained as described (Ashburner, *Drosophila. A Laboratory Manual*, Plainview NY: cold Spring Harbor Laboratory Press (1989)). Larval CNSs were dissected in Ringer's solution, fixed in 30%

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glutaraldehyde and X-gal stained as described (Ashburner, *Drosophila. A Laboratory Manual*, Plainview NY: Cold Spring Harbor Laboratory Press (1989)) for one hour at 37°C. Adult heads were imbedded in OCT, sectioned in a cryostat, fixed in 1% glutaraldehyde/PBS, X-gal stained and mounted as described by Han et al. *Neuron* 9:619-627 (1992)).

Behavioral Analysis of Transgenic Flies

Preparation of flies

Before behavioral assays, approximately 600 1-to-2-day old flies were placed in a foam-plugged 1/2 pint glass bottle with standard food and a wad of paper towel. These flies were stored overnight at 25°C and 50% relative humidity. The next morning, groups that were destined for the heat-shock treatment (37°C for 30-minutes) were transferred to foam-plugged, 15 x 85 mm glass vials with a 10 x 20 mm strip of Whatman 3M filter paper. The vials were placed in a water bath, ensuring that the fly chamber was completely submerged. After this heat-shock regimen, the flies were transferred to a standard food vial where they recovered for three hours at 25°C and 50% relative humidity, at which time behavioral assays commenced.

Pavlovian Learning/Memory

To analyze associative learning, the Pavlovian conditioning procedure of Tully and Quinn (*J. Comp. Physiol. A.* 157:263-277 (1985)) was used. Briefly, groups of about 100 flies were trained in a tube with an internal electrifiable grid. The tube was sequentially ventilated with two odorants, 3-octanol (OCT; ICN Biochemical, Aurora OH) and 4-methylcyclohexanol (MCH; Fluka Chemie AG, Buchs CH) at concentrations equally aversive to untrained flies. The flies were exposed for 60 seconds to OCT (CS+), while being given twelve 1.5-s pulses of 60V (DC) electroshock every 5 seconds,

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followed by a 45-second rest period. The flies then were exposed for 60 seconds to MCH (CS-) without any electroshock, which again was followed with a 45-s rest interval. To test for learning, the trained flies were

5 tapped into a T-maze immediately after this discriminative conditioning procedure. Air laced with the CS+ or CS- was drawn through each of the two respective arms of the T-maze, and the flies were allowed 120 seconds to migrate into either t-maze arm.

10 At the end of this test trial, the flies were trapped in the T-maze arms, anesthetized and counted. For one complete experiment, this training/testing procedure was repeated with a second group of flies using the reciprocal odor combination (MCH as CS+ and OCT as CS-).

15 The total numbers of flies in the T-maze arms then were used to calculate the proportions "correctly avoiding" the CS+ (they were in the CS- T-maze arm), and the two values from reciprocal experiments were averaged. Finally, a performance index (PI) for one complete

20 experiment was calculated by normalizing the average proportion "correctly avoiding." PIs could range from 0 (a 50:50 distribution in the T-maze; no learning) to 100 (all flies avoid the CS+). To measure 3-hour retention, trained flies were transferred to a food vial, where

25 they were stored at 25°C during the retention interval. Seventy-five seconds before the test trial, flies were transferred to the choice point of the T-maze and tested as described above.

Olfactory Acuity

30 The flies' ability to smell the odorants used during Pavlovian conditioning experiments was quantified by exposing groups of untrained flies for 120-seconds to odor vs. air in the T-maze (see Boynton and Tully, *Genetics* 131:655-672 (1992)). Typically, two odor

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concentrations were used: undiluted, as in the Pavlovian experiments, and a 100-fold dilution. Performance indices (PIs) were calculated as above but for each group separately. To control for "side bias," equal numbers of groups were assayed with odor in the right arm, or odor in the left arm, of the T-maze.

Shock Reactivity

The flies' ability to sense the electroshock used during Pavlovian conditioning experiments was quantified by introducing groups of untrained flies into a testing T-maze where both arms contain electrifiable grids. One of the two arms were electrified as above, and the flies chose between shock vs. no shock for 120 seconds (see Luo et al. *Neuron* 9:595-605 (1992); the primary reference to this method was incorrectly stated as Dura et al. *J. Neurogenet.* 9:1-14 (1993) in Tully et al., *Cell* 79:35-47 (1994); Yin et al., *Cell* 79:49-58 (1994); Yin et al., *Cell* 81:107-115 (1995)). Typically, two shock voltages were used: 60V, as in the Pavlovian experiments, and 20V. Performance indices (PIs) were calculated as in olfactory acuity experiments.

Statistical Analysis of Behavioral Data

PIs are distributed normally (Tully and Gold, *J. Neurogenet.* 9:55-71 (1993)), so untransformed data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). All pairwise comparisons were planned. To maintain an experiment wise error rate of $\alpha=0.05$, the critical P values were adjusted accordingly (Sokal and Rohlf, *Biometry* (New York, Freeman 1981)); Audesirik and Audesirik, *NeuroToxicology* 10:659-670 (1989)) and are listed below for each experiment. All behavioral experiments were performed in a balanced fashion, with N=2 PIs collected per day per group (genotype \pm hs). In these experiments, the experimenter was blind to genotype.

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Learning in Wild-type (Canton-S), Mutant (*lio*¹) and Transgenic (*lio*¹;*hslio*⁻¹⁶ or *lio*¹;*hslio*⁻³) Flies With and Without Heat Shock

PIs from four genotypes (wild-type, *lio*¹, *lio*¹;*hslio*⁻¹⁶ or *lio*¹;*hslio*⁻³) and two heat shock regimens (-hs or +hs) were subjected to a two-way ANOVA with geno ($F_{(3,72)}=71.30$, $P<0.001$) and heat shock regimen ($F_{(1,72)}=17.74$, $P<0.001$) as main effects and geno x heat ($F_{(3,72)}=21.68$, $P<0.001$) as an interaction term. In the absence of heat-shock, learning in *lio*¹ mutants was significantly lower than in wild-type flies ($P<0.001$), while learning in transgenic *lio*¹;*hslio*⁻¹⁶ and *lio*¹;*hslio*⁻³ flies was similar to that in *lio*¹ mutants ($P=0.015$ and $P=0.87$, respectively). When trained three hours after heat-shock, learning in *lio*¹ mutants still was significantly lower than that in wild-type flies ($P<0.001$), but learning in transgenic *lio*¹;*hslio*⁻¹⁶ and *lio*¹;*hslio*⁻³ flies was significantly improved ($P<0.001$ for each) and did not differ from wild-type flies ($P=0.071$ and $P=0.22$, respectively). In contrast to this clear effect of heat shock on learning in transgenic flies, heat shock had no effect on learning in wild-type flies ($P=0.006$) or in *lio*¹ mutants ($P=0.084$). The ten planned comparisons were deemed significant if $P\leq 0.005$.

Three-hour Retention in Wild-type (Canton-S), Mutant (*lio*¹) and Transgenic (*lio*¹;*hslio*⁻¹⁶ or *lio*¹;*hslio*⁻³) Flies With or Without Heat Shock

PIs from four genotypes (wild-type, *lio*¹, *lio*¹;*hslio*⁻¹⁶ or *lio*¹;*hslio*⁻³) and two heat shock regimens (-hs or +hs) were subjected to a two-way ANOVA with geno ($F_{(3,72)}=20.54$, $P<0.001$) and heat shock regimen ($F_{(1,72)}=20.49$, $P<0.001$) as main effects and geno x heat ($F_{(3,72)}=7.67$, $P<0.001$) as an interaction term. The ten planned comparisons were deemed significant if $P\leq 0.005$. As was true for learning, three-hour retention in *lio*¹ mutants was significantly lower than in wild-type flies

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($P < 0.001$) in the absence of heat-shock, while learning in transgenic *lio¹;hsl¹⁰-16* and *lio¹;hsl¹⁰-3* flies was similar to that in *lio¹* mutants ($P = 0.76$ and $P = 0.91$, respectively). When trained three hours after heat-shock, three-hour retention in transgenic *lio¹;hsl¹⁰-16* and *lio¹;hsl¹⁰-3* flies was significantly improved ($P < 0.001$ for each) and did not differ from wild-type flies ($P = 0.67$ and $P = 0.69$, respectively). In contrast to this clear effect of heat shock on three-hour retention in transgenic flies, heat shock had no effect on three-hour retention in wild-type flies ($P = 0.763$) or in *lio¹* mutants ($P = 0.93$).

Olfactory Acuity in Wild-type (Canton-S) and Transgenic (*lio¹;hsl¹⁰-16*) Flies

PIs from two genotypes (wild-type or *lio¹;hsl¹⁰-16*), four odor/concentration groups, (OCT 10^{-2} ; OCT 10^0 ; MCH 10^{-2} or MCH 10^0) and two heat shock regimens (-hs or +hs) were subjected to a three-way ANOVA with genotype ($F_{(1,128)} = 0.29$, $P = 0.59$), odor ($F_{(3,128)} = 61.76$, $P < 0.001$) and heat shock regimen ($F_{(1,128)} = 33.33$, $P < 0.001$) as main effects, with geno x odor ($F_{(3,128)} = 1.79$, $P = 0.15$), geno x heat ($F_{(1,128)} = 0.04$, $P = 0.84$) and odor X heat ($F_{(3,128)} = 0.15$, $P = 0.93$) as two-way interaction terms and with geno x odor x heat ($F_{(3,128)} = 0.15$, $P = 0.93$) as the three-way interaction term. The twelve planned comparisons were judged significant if $P \leq 0.004$ and are summarized in Table 1.

Shock Reactivity in Wild-type (Canton-S) and Transgenic (*lio¹;hsl¹⁰-16*) Flies

PIs from two genotypes (wild-type or *lio¹;hsl¹⁰-16*), two voltages (20V or 60V) and two heat shock regimens (-hs or +hs) were subjected to a three-way ANOVA with geno ($F_{(1,64)} = 0.57$, $P = 0.45$), volt ($F_{(1,64)} = 97.47$, $P < 0.001$) and heat shock regimen ($F_{(1,64)} = 0.14$, $P = 0.71$) as main effects, with geno x volt ($F_{(1,64)} = 0.63$, $P = 0.43$), geno

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x heat ($F_{(1,64)}=0.21$, $P=0.65$) and volt x heat ($F_{(1,64)}=2.81$, $P=0.10$) as two-way interaction terms and with geno x volt x heat ($F_{(1,64)}=0.63$, $P=0.43$) as the three-way interaction term. The six planned comparisons were
5 judged significant if $P \leq 0.01$ and are summarized in Table 1.

Learning in Wild-type (Canton-S) and Transgenic (*lio⁺;hslio⁻-16*) Flies With or Without Heat Shock

PIs from two genotypes (wild-type or *lio⁺;hslio⁻-16*)
10 and two heat shock regimens (-hs or +hs) were subjected to a two-way ANOVA with genotype ($F_{(1,20)}=0.02$, $P=0.89$) and heat shock regimen ($F_{(1,20)}=36.04$, $P<0.001$) as main effects and geno x heat ($F_{(1,20)}=0.39$, $P=0.54$) as an interaction
term. Conditioned odor avoidance after olfactory
15 learning was quantified as described above. $N=6$ PIs per group. The two planned comparisons were deemed significant if $P \leq 0.05$, and results are summarized in Figure 4A. Learning in transgenic flies did not differ significantly from wild-type flies in the absence of
20 ($P=0.60$), or when trained three hours after ($P=0.73$), heat-shock.

Three-hour Retention in Wild-type (Canton S) and Transgenic (*lio⁺;hslio⁻-16*) Flies With or Without Heat Shock

PIs from two genotypes (wild-type or *lio⁺;hslio⁻-16*) and two heat shock regimens (-hs or +hs) were
25 subjected to a two-way ANOVA with geno ($F_{(1,20)}=1.27$, $P=0.27$) and heat shock regimen ($F_{(1,20)}=3.37$, $P=0.08$) as main effects and geno x heat ($F_{(1,20)}=0.01$, $P=0.93$) as an
30 interaction term. The two planned comparisons were deemed significant if $P \leq 0.05$ and are summarized in Figure 4B. As was true for learning, three-hour retention in transgenic flies did not differ significantly from wild-type flies in the absence of

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($P=0.47$), or when trained three hours after ($P=0.40$), heat-shock.

Statistical Analysis of Histological Data

Planimetric estimates of neuropilar volume of mushroom bodies (sum of both hemispheres) and central complex were distributed normally, so raw data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). Data from each anatomical region were subjected separately to a two-way ANOVA with geno [$(F_{(3,64)}=1.49, P=0.23)$ for central complex; $(F_{(3,64)}=0.77, P=0.52)$ for mushroom bodies] and sex [$(F_{(1,64)}=172.13, P<0.001)$ for central complex; $(F_{(1,64)}=38.68, P<0.001)$ for mushroom bodies] as main effects and geno x sex [$(F_{(3,64)}=8.47, P<0.001)$ for complex; $(F_{(3,64)}=6.21, P<0.001)$ for mushroom bodies] as an interaction term. To maintain an experiment wise error rate of $\alpha=0.05$, critical P values for the four planned comparisons (Figure 6) were adjusted to $P\leq 0.01$. Experimenters were blind to genotype during histological preparations of tissue sections and during planimetric analyses. No significant differences between ++ versus *lio¹/lio¹* or *+/Df* versus *+/lio¹* flies were detected in males or females for either anatomical region for N=9 flies per genotype for males and 6, 12, 12 and, N=6 files for *+/+*, *+/Df*, *lio¹/Df* and *lio¹/lio¹* females, respectively.

Characterization of the Genomic Region Surrounding the *linotte* P Element Insertion

Tagging the *linotte* gene with a *PlacW* transposon allowed its immediate localization *in situ* to cytological region 37D on the left arm of the second chromosome using P element DNA as a probe (Dura et al. *J. Neurogenet.* 9:1-14 (1993)). The *PlacW* transposon also contains an origin of DNA replication and ampicillin resistance gene, which allowed the direct

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cloning of a 900 bp fragment of genomic DNA flanking the *linotte*¹ (*lio*¹) P-element insertion as a bacterial plasmid (see Figure 1).

This 900 bp genomic DNA fragment was used to probe
5 a Southern blot of wild-type (Canton-S) genomic DNA.
The 900 bp probe appeared to be non-repetitive and
subsequently was used to screen a *Drosophila* genomic DNA
bacteriophage λ library (kindly provided by R.L. Davis).
A total of eight genomic clones were recovered;
10 restriction mapping indicated that these covered a 24 kb
genomic region (Figure 1). Cross-hybridization of the
900 bp "rescue fragment" with restriction fragments of
the λ clones indicated that the *linotte* P element was
inserted in a 1kb EcoRI-HindIII fragment (Figure 1).

15 The 900 bp genomic fragment also hybridized *in situ* to
chromosomal region 37D (data not shown), thereby
verifying that the appropriate flanking DNA was cloned.

Cytological localization of the *lio*¹ P element
insertion placed it just proximal to the *Dopa*
20 *decarboxylase* (*Ddc*) gene (37C; Hirsh and Davidson, *Mol.*
Cell. Biol. 1:475-485 (1981)). Approximately 180 kb of
the genomic region surrounding *Ddc* had already been
cloned by Stathakis et al., *Genetics* (submitted) (1995),
so the *lio*¹ and *Ddc* genomic regions were compared. The
25 most proximal *Ddc* genomic clone (λ CS2.27) cross-
hybridized with all our λ clones on a Southern blot and
contained overlapping restriction maps (Figure 1). This
placed the *lio*¹ P element insertion approximately 65 kb
proximal to the *Ddc* locus.

30 Identification of RNA Transcripts in the *linotte* Genomic Region

To identify RNA transcripts near the *lio*¹ P element
insertion, subcloned restriction fragments from the
genomic λ clones were used initially to probe Northern
35 blots of wild-type (Canton-S) adult whole-fly polyA+
RNA. A 5.7 kb Hind III fragment distal to the P element

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insertion (see Figure 1) hybridized to a 3.5 kb and a 1.9 kb RNA species (data not shown). This 5.7 kb genomic fragment then was used to screen a Canton-S adult head cDNA library (kindly provided by R.L. Davis).

5 Five cDNA clones were identified; cDNA restriction mapping and Southern and Northern blot analyses revealed that these clones fell into two non-crosshybridizing classes. One cDNA class hybridized only to the 1.9 kb RNA transcript and to the 1.2 kb HindIII-SacII genomic

10 fragment (Figure 1). By these criteria and via direct sequencing of one of the cDNA clones (data not shown), this cDNA class was determined to correspond to the female sterility gene, *fs(2)TW1* (see Stathakis et al., Genetics submitted (1995). Female fertility appeared

15 normal in the original *lio*¹ mutants and in over 100 lines homozygous for independent excisions of the *lio*¹ P element insertion. Thus, *fs(2)TW1* is not likely to correspond to the *linotte* gene.

The second cDNA class hybridized only to the 3.5 kb

20 RNA transcript and to the 0.9 kb SacII-HindIII genomic fragment situated just 800 bp distal to the *lio*¹ P element insertion (Figure 1). A 3.1 kb cDNA clone of this class was used as a probe on Northern blots from two independent extractions of polyA⁺ RNA from wild-type

25 and *lio*¹ P element insertion (Figure 1). A 3.1 kb cDNA clone of this class was used as a probe on Northern blots from two independent extractions of polyA⁺ RNA from wild-type and *lio*¹

adult heads, revealing in *lio*¹ mutants 54±2% of normal

30 levels of the 3.5 kb transcript (see Examples below). The 3.5 kb transcript was detected with only one strand-specific probe from the 3.1 kb cDNA (data not shown), thereby indicating the direction of transcription. Such a Northern blot was also probed with a 6 kb EcoRI

35 genomic restriction fragment (Figure 1) just proximal to the *lio*¹ P element insertion. No transcripts were detected (data not shown).

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Taken together, these data suggested that the *linotte* gene encodes the 3.5 kb transcript. The *linotte* P element insertion is approximately 300 bp proximal to this transcript and the *fs(2)TW1* transcript lies immediately distal to this putative *linotte* transcript (Stathakis et al. Genetics (submitted) (1995). The *lio*¹ P element does not appear to be inserted in the transcription unit itself but nevertheless reduces its level of expression, thereby suggesting the *lio*¹ mutation to be hypomorphic. Previously published genetic data (Dura et al., J. Neurogenet. 9:1-4 (1993)), in contrast, have suggested that the *lio*¹ mutation is amorphic. Resolution to this apparent discrepancy must await further investigations of the effect of various levels of Lio activity on learning and of the spatial distribution of Lio in adult heads.

Induced Expression of a *hslio*⁺ Transgene Rescues the *linotte* Mutant Learning and Memory Deficits

Since the *linotte* mutant strain originally was isolated from a transposon-mediated mutagenesis, this foreign piece of DNA might influence the expression of more than one gene in the region. Thus, no molecular or histological data were sufficient to identify the transcript associated with the *linotte* learning defect. Correct identification of the *lio* transcript was obtained, however via transgenic rescue of the *linotte* learning deficit. The 3.1 kb cDNA clone was fused to the *heat shock-70* (*hs-70*) promoter sequence to construct an inducible *hslio*⁺ minigene. Several transgenic lines carrying independent genomic insertions of this *hslio*⁺ construct were generated first on a *lio*⁺ (wild-type) background. Then the *hslio*⁺ insertions were crossed into *lio*¹ mutants.

Mutant *linotte* flies originally were isolated because of a 3-hour memory retention deficit but subsequently were shown to have impaired learning as

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well (Dura et al., *J. Neurogenet.* 9:1-14 (1993)). Thus, both learning and 3-hour retention were assayed in *hsllo'* transgenic flies. Our previous experiments have shown robust heat-shock induction of *hs-70* promoter-driven transgenes with minimal non-specific effects on learning and memory formation by exposing adults flies to one, 30-minute heat shock (37°C) three hours before training (Yin et al., *Cell* 79:49-58 (1994); Yin et al., *Cell* 81:107-115 (1995a)). This heat shock regimen was used here.

In the absence of heat shock, two *hsllo'* transgenic lines (*lio'*; *hsllo'+-3* and *lio'*; *hsllo'-16*) displayed learning and 3-hour retention scores similar to those of the *lio'* mutant (Figures 3A and 3B). After heat shock-induction of the *hsllo'* transgenes, however, learning and 3-hour retention scores were similar to those of wild-type flies. In contrast, this heat shock regimen had no effect on the learning or 3-hour retention scores of wild-type flies or *lio'* mutants lacking a transgene. Learning also was assayed in two other transgenic lines (*lio'*; *hsllo'-1* or *lio'*; *hsllo'-21*). Mean (PI \pm SEM) scores for *lio'*; *hsllo'-21* transgenic flies were 63 \pm 3 (N=6) in the absence of heat shock and 80 \pm 2 (N=6) three hours after heat shock, while those for *lio'*; *hsllo'-1* were 50 \pm 6 (N=2) in the absence of heat shock and 61 \pm 2 (N=2) three hours after heat shock. Thus, while results from *lio'*; *hsllo'-21* transgenic flies were similar to those of *lio'*; *hsllo'+-3* and *lio'*; *hsllo'-16* transgenic flies, induced expression of the transgene in *lio'*; *hsllo'-1* flies appeared to yield intermediate results.

Consistent with the apparent behavioral rescue of the *linotte* mutation by induced expression of the *hsllo'* transgene, a Northern blot analysis on polyA+RNA from adult *lio'*; *hsllo'-16* heads showed an increased level of expression of the *hsllo'* transcript three hours after heat shock induction, while levels of expression of the endogenous *lio'* transcript in wild-type, *lio'*, or *lio'*;

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hsl10⁻-16 flies remained unchanged. The transgenic transcript was undetectable in the absence of heat shock, indicating little leaky expression of the transgene and was again undetectable six hours after
5 heat shock. This same heat shock regimen induces high levels of expression of the *hsdCREB2-b* or *hs-dCREB2-a* transgenic transcripts, and they, in contrast, then remain detectable for more than nine hours (Yin et al., Cell 79:49-58 (1994); Yin et al., Cell 81:107-115 (1995
10 a)). Thus, turnover of the *hsl10⁻* transcript appears to be relatively rapid.

Induced Expression of the *hsl10⁻* Transgene Does Not Affect Olfactory Acuity or Shock Reactivity

To understand the effects of single-gene mutations
15 on learning/memory, it has been argued that poor performance in learning/memory assays cannot be interpreted properly without also assessing the task-relevant sensory/motor responses evoked in untrained animals by the stimuli used in the learning/memory
20 procedures (Gailey et al., J. Comp. Physiol. A. 169:685-697 (1991); Boynton and Tully, Genetics 131:655-672 (1992); Luo et al., Neuron. 9:595-605 (1992); Dura et al., J. Neurogenet. 9:1-14 (1993); Tully et al., Cell 79:35-47 (1994); Yin et al., Cell 79:49-58 (1994);
25 Mihalek et al., (submitted 1995); Yin et al., Cell 81:107-115 (1995)). As described herein, assays of olfactory acuity and shock reactivity have been developed, which quantify the abilities of the flies to sense the same odors and electroshock in the same T-maze
30 apparatus used for assays of Pavlovian learning/memory. For this study, olfactory acuity and shock reactivity were assayed in untrained flies in the absence of, and three hours after, the usual heat shock regimen. This post-heat shock time point was chosen to correspond to
35 the time when flies were trained in the Pavlovian learning experiments.

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Table 1 lists the olfactory acuity and shock reactivity scores from wild-type and transgenic (*lio*¹; *hsl**lio*⁻¹⁶) flies with or without the heat shock treatment (\pm hours) and from mutant *lio*¹ flies without heat shock.

5 In the absence of heat shock, olfactory acuity and shock reactivity mean scores (PI \pm SEM) were similar among wild-type, *lio*¹; *hsl**lio*⁻¹⁶ and *lio*¹ flies (cf. Dura et al., *J. Neurogenet.* 9:1-14 (1993)). This observation indicates that the genetic backgrounds of the three strains were

10 similar. Three hours after heat shock, olfactory acuity and shock reactivity mean scores still did not differ between wild-type and *lio*¹; *hsl**lio*⁻¹⁶ flies. In light of these data, the behavioral rescue of mutant *lio*¹ flies by induced expression of the *hsl**lio*⁻ transgene observed in

15 Pavlovian learning/memory experiments (see above) now can be interpreted as a specific rescue of learning/memory per se.

Table 1. Olfactory acuity and shock reactivity in wild-type (Canton-S), mutant *lio*¹ and transgenic *lio*¹; *hsl**lio*⁻¹⁶ flies.

Heat Shock	Group	Olfactory Acuity ¹				Shock Reactivity	
		OCT		MCH			
		10 ⁰	10 ⁻²	10 ⁰	10 ⁻²	60V	20V
-hs	wild-type <i>lio</i> ¹	74 \pm 3	35 \pm 7	85 \pm 3	42 \pm 7	81 \pm 5	42 \pm 10
		82 \pm 3	42 \pm 5	81 \pm 2	35 \pm 7	74 \pm 4	54 \pm 4
-hs	wild-type <i>lio</i> ¹ ; <i>hsl</i> <i>lio</i> ⁻¹⁶	69 \pm 5	44 \pm 6	72 \pm 3	33 \pm 4	87 \pm 2	62 \pm 4
		75 \pm 4	43 \pm 6	68 \pm 4	38 \pm 5	84 \pm 3	59 \pm 6
-hs ²	wild-type <i>lio</i> ¹ ; <i>hsl</i> <i>lio</i> ⁻¹⁶	52 \pm 6	31 \pm 3	62 \pm 4	21 \pm 3	87 \pm 3	57 \pm 7
		60 \pm 6	32 \pm 5	52 \pm 3	25 \pm 5	92 \pm 1	51 \pm 6

¹ Olfactory acuity and shock reactivity were assayed in untrained flies with the methods of Boynton and Tully *Genetics* 131:655-672 (1992) and Luo et al. *Neuron* 9:595-605 (1992), respectively (see Experimental Procedures). N=8 PIs per group. Planned comparisons between wild-type vs. mutant flies failed to detect any significant differences. 10⁰ corresponds to undiluted odorants, 10⁻² is a 100-fold dilution in mineral oil.

² The heat shock regiment was identical to that used for Pavlovian learning/memory assays; flies were assayed 3 hours post heat shock.

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Induced Expression of the *hslio*⁺ Transgene Does Not Affect Learning or Memory of Wild-type Flies

The issue of whether the improved performance of induced *lio*¹; *hslio*⁺-16 transgenic flies reflects a
5 general enhancement of learning/memory or rather a specific rescue of the *linotte* mutation was addressed by studying the *hslio*⁺-16 transgenic insertion in a *lio*⁺ (wild-type) background rather than the *lio*¹ mutant background. In this manner, the effects on learning and
10 memory of induced (and ectopic) overexpression of the *lio*⁺ transgene were quantified (Figures 4A and 4B).

In the absence of heat shock, mean learning or 3-hour retention scores (PI±SEM) did not differ between wild-type and transgenic *lio*⁺; *hslio*⁺-16 flies, again
15 indicating that genetic backgrounds of the two strains were similar. When trained three hours after heat shock, mean learning and 3-hour retention scores between these two strains still did not differ. These data demonstrate that induced overexpression of the *lio*⁺
20 product does not enhance learning or memory generally. Thus, it can be concluded that induced rescue of the learning/memory deficit in *lio*¹; *hslio*⁺ transgenic flies (see above) represents a specific rescue of the *lio*¹ mutation.

25 The *linotte* Transcript Encodes a Novel Protein

Full rescue of the *lio*¹ learning/memory deficits by induced expression of a *hslio*⁺ transgene constitutes definitive proof that the correct RNA transcript had been identified. Thus, a closer (molecular) look at the
30 corresponding 3.1 kb cDNA clone was warranted. Sequence analysis of the 3.1 kb cDNA revealed one prominent 2.7 kb translational open reading frame (ORF) in the transcribed orientation (Figure 5A). The transcribed orientation of the *lio*⁺ cDNA (SEQ ID NO: 1) was
35 determined by strand-specific probing of a Northern blot

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of whole-fly Can-S polyA+RNA. Several stop codons occurred 5' of this putative ORF, and the nucleotides immediately preceding the translational start site conformed with the *Drosophila* (and general) conservation rules for active translational start sites (Cavener, *Nucleic Acids Res.* 15:1353-1361 (1987); Kozak, *Nucl. Acids. Res.* 15:8125-8148 (1987)). Only one reading frame yields a deduced amino acid sequence of appreciable length. This ORF initiates 89 nt from the 5' end of the 3.1 kb cDNA, extends 2748 nt and terminates 262 nt from the 3' end.

This ORF encodes a 916 amino acid, 103kD deduced polypeptide (SEQ ID NO: 2) and terminates 800 nt upstream of the 3' end of the cDNA sequence. The deduced amino acid sequence (Figure 5B, SEQ ID NO: 2) bears no significant homology to any previously characterized protein in the BLAST, Swiss-Protein or Pro-Site databases. The *linotte* gene, therefore, appears to encode a novel protein involved in associative learning.

The *lio*⁺ Transcript is Detected in Embryos, Pupae and Adults But Not Larvae

To investigate the developmental expression of the *linotte* transcript, the 3.1 kb cDNA was used in Northern blot analyses to probe polyA+RNA from various developmental stages. At all stages, only one 3.5 kb transcript was detected. Levels of expression of this message differed significantly during development. The *lio*⁺ mRNA was expressed at a high level in early embryos (0-4 hours) but not in late embryos (>16 hours), suggesting a maternal origin for the early signal. The *lio*⁺ message was not detected during the larval stages but then reappeared during pupal development and was expressed at high levels in adult head and body.

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The Developmental Pattern of Expression of the Enhancer-trap Reporter Gene in *lio*¹ Mutants Does Not Correspond to that of the *lio*¹ Transcript

The *lio*¹ mutation resulted from the insertion of a transposable enhancer-trap P element (*PlacW*) containing a *lacZ* reporter gene, which could be activated transcriptionally by regional enhancer elements (Bier et al., *Genes Dev.* 3:1273-1287 (1989)). New mutant alleles of *rutabaga*, for instance, were recovered with *PlacW* insertions in the 5' untranslated region of the gene (Levin et al., *Cell* 68:479-489 (1992)). The pattern of *lacZ* reporter gene expression in these mutants overlapped extensively with *rut* protein expression (Han et al., *Neuron* 9:619-627 (1992)).

To observe *lacZ* reporter gene activity in the CNS of *linotte* mutants, embryos, third-instar larvae and adults were stained with the chromogenic *lacZ* substrate, X-gal. *lacZ* activity in a whole-mount stage 14 embryo (100X) was detected in the brain and in the ventral nerve cord. *lacZ* activity also was detected in the peripheral nervous system and in the posterior midgut. In whole-mount, dissected third-instar larval CNSs (200X), enhancer trap-driven expression of the *lacZ* reporter gene was appreciable in the dorsal-medial region of the brain, in the lateral brain hemispheres, the developing adult visual system and in the ventral ganglia. In 10µm frontal sections of adult heads (200X), enhancer trap-driven expression of the *lacZ* reporter gene was observed in the dorsal-medial region, in the optic lobes and in the subesophageal ganglion. No staining was apparent in cells surrounding the mushroom body calyces (data not shown).

In stage 12 embryos *lacZ* reporter gene activity was detected in the central and peripheral nervous systems and in several other locations. In third-instar larvae, a high level of *lacZ* reporter gene activity was observed in the lateral hemispheres of the brain lobes, where the

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adult visual system develops, and to a lesser degree in the dorsal medial region of the brain and in the ventral ganglia. In frontal sections of adult heads, *lacZ* reporter gene activity also was observed in a few
5 neurons in the dorsal medial region of the protocerebrum and in the optic lobes and subesophageal ganglion. No prominent *lacZ* activity was detected in the calyces of the mushroom bodies, where the *dunce* and *rutabaga* genes are preferentially expressed (Nighorn et al., *Neuron*
10 6:455-467 (1991); Han et al., *Neuron* 9:619-627 (1992)). Notably, this developmental pattern of *lacZ* expression does not coincide with the temporal pattern of *lio'* expression (data not shown).

Gross Anatomy of Mushroom Bodies and Central Complex is
15 Normal in *linotte* Mutants

Genetic or chemical lesions of two anatomical regions of the adult brain, the mushroom bodies and the central complex, disrupt olfactory learning (Heisenberg et al., *J. Neurogenet* 2:1-30 (1985); de Belle and
20 Heisenberg, *Science* 263:692-695 (1994)). Subtler aspects of mushroom body development also are defective in *dunce* and *rutabaga* mutants (Balling, *J. Neurogenetics* 4:65-73 (1987)), and defects in the central complex, in fact, have been reported for *linotte* mutants (Dura et
25 al., *J. Neurogenet* 10:25 (1995)).

Full rescue of the *lio'* learning/memory deficit by induced expression of the *hs-lio'* transgene in adults (see above) brought into question the latter claim, however. Thus, adult brain structure in *lio'* mutants
30 were reassessed. Mushroom bodies and central complex were visually inspected, and their neuropilar volumes were quantified via planimetric analysis in wild-type (*lio'*) flies, *lio'* homozygous mutants and hemizygous flies carrying either the wild-type (*lio'*) or *lio'*
35 chromosome and a second chromosome deletion (*Df*, see Experimental Procedures) of the *linotte* region. (The

dosages of *lio*⁺ or *lio*⁻ in these hemizygous flies were only 50% of those in corresponding homozygous flies, thereby potentially yielding more severe phenotypic defects). In a double-blind experiment, frontal
5 sections of *lio*¹/*lio*¹, *lio*¹/*Df*, *lio*⁺/*Df* and *lio*⁺/*lio*⁺ heads were serially sectioned in the laboratory of Dr. M. Heisenberg, and then planimetric analyses of mushroom bodies and central complex were carried out in our laboratory. The different preparations were fixed,
10 embedded in paraffin, cut into 7 μ m sections and inspected by fluorescence microscopy (400X). These analyses failed to detect any qualitative or quantitative differences among the four genotypes in these two brain structures (data not shown).

15 Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such
20 equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

We claim:

1. A gene present in cytological region 37D of the second chromosome which functions in associative learning, said gene comprising the DNA sequence of SEQ ID NO: 1.
2. A gene which functions in associative learning and which hybridizes under standard conditions to the DNA sequence of SEQ ID NO: 1 or the complement of said DNA sequence.
3. A gene according to Claim 1, wherein disruption of said gene results in decreased associative learning and/or memory.
4. A protein encoded by the gene of Claim 1.
5. A protein encoded by the gene of Claim 2.
6. A protein according to Claim 4 comprising the amino acid sequence of SEQ ID NO: 2.
7. An antibody which binds to the protein of Claim 4.
8. An antibody which binds to the protein of Claim 5.
9. A nucleic acid sequence comprising the nucleic acid sequence of SEQ ID NO: 1 or the complement of said nucleic acid sequence.
10. An amino acid sequence comprising the amino acid sequence of SEQ ID NO: 2.
11. DNA encoding the amino acid sequence of SEQ ID NO:2.

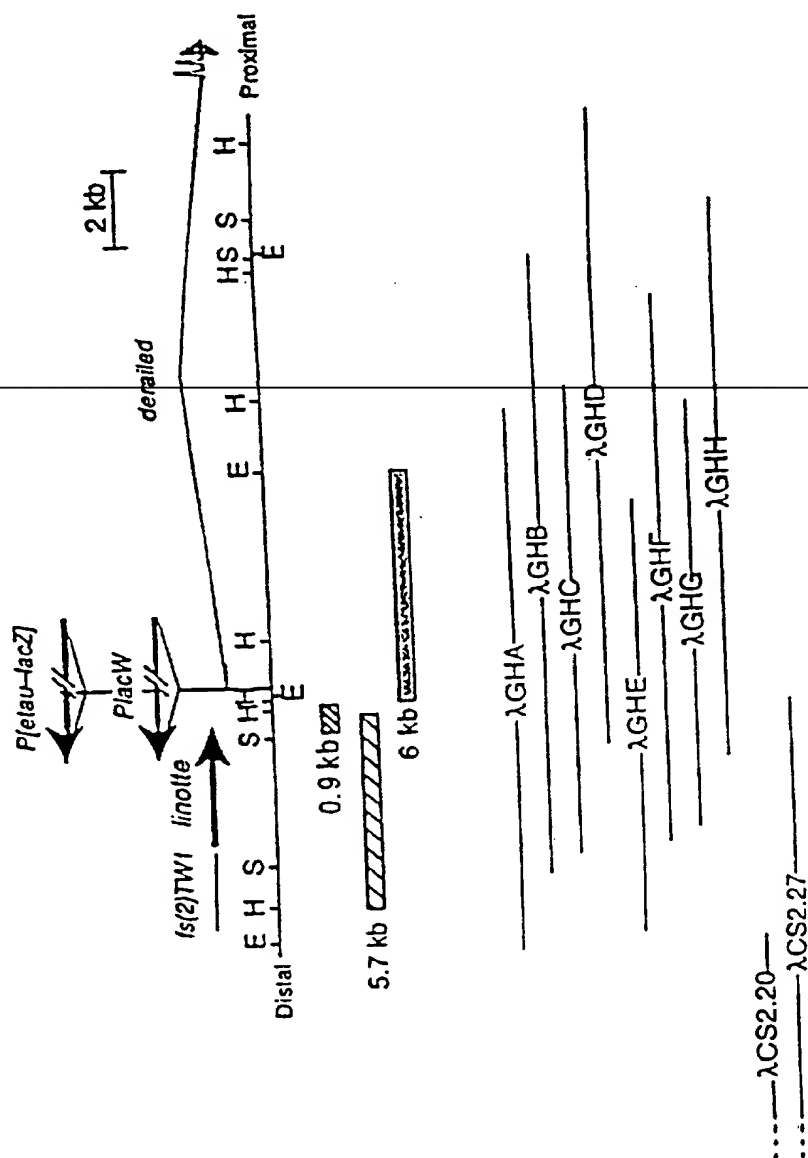


Fig. 1

Fig. 2

CTGAGAAGCGCGCAACGTGCACACTGGCAGGCTGATTGAAAAAATTCGTTGCAAATGTTT
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CTGCGGTCTCCTGGCCAGCCAGGGCTATTAAGAGAAGGCAAACGAGTGGCGCATTTTGGG
CCAGGAACAGGATGGATCTCTGCTCACGTCCTGGATATTCGAGTACGCGGACGAGGATCA
GCGCAAGGAGACGTGCATTGGCCACTTTCACGCCACCAAGAAGCAGCTGCGACTCCTTTG
GACCCTCGACAATTGCGGTGAGATCGTCCAGGCAACGATTAACAGCAGTGTACATTGCT
GTCCTTCGTGGAGAAAACAGGGCAAGCTCTATCAGGCCCTTTGTCTGGAGGTAAGGAG
CTCCGAAGGTGGCACGGCCACGCCCTCAACTCGGAGCCCTCCAACCGCCAGATGATGAC
GCAGTTCTGTGGCGCGTCCGAGAGTGCACGCGCACCTGCTGGCAGGACAAGCTACTGGT
GCTCACCCACGAGGAATCCATCAAGCAGTACAGCTGCGTGGTCAAGCAGAGCTCCACCAC
ATGCTCGACTGGCGGAGGCGAGGGCAGCGCTAGAGGCTAGACACCAGCATACTGACCTA
CGAAACGCTGGCCAGGAACCTTAGCTGGGCCAGTGGGATCCCGAGTGGCAGGCTCTTTA
TTACATTCACTTGAAGCCGAAGGCCAAGAGCCTCAGTCTGCTGGACGAGAGGGAGGAGGC
TGGCGAGCAGACAACCTCTACTTTAAGCCCCACGCTCTCGGCCCTTCAGTTTAAAGAAA
ACAGCCAAACGGAACAGTGTCTAATATACCCCTCAATTTGCCAAAGCTGCCCAATGGCTC
CAAAGAGGAATCGCCAAGCTACGATGACGATGCGGTTCCCTTGGCGGTGCACGATAGTTC
GCTAAATCTCATCATACTGGCGGACACCTCGGGCATGTTTTCGTCTGTCACTACTACTCT
GTACCAGCCGATGCAGTGGAGCAAAGGGATGTGCACTTTGCCCTACTCGGTGACTTTGCT
TCACCACGGCTGTGTGGTGCACTGCGTCAATGCCCGGTGTGCCGTGGCAAAGGCCCGTCT
GCTGAGGCCAACATTTGCGCTACACGCCAGCATCACTGCTGGTGTGCTCCGCCCTTTTT
TGTCACCTCTTGGACGTGGGACTGCAGCACGAACCGAAGTGCATATCGTGTGTGCAGC
CCACAATCGAAGTCCCGATATCAACAGCTGGTGCCTTTGCGAAAGTGGGGAGCTCTGGC
TTATGATGCGGCTACCTTGGATCTGGTCTCGTTGTCCGTGCCCAAATCCCATTTGATAGA
GGCTTTCCGCAATGACAGTTCCGCTGGACAATAGAATCAGCATTATCCACTACTTCCTTTT
CGACTCGAAGCATATGGATGTGTGGCCGAGCTGCTGAACAAATATCTTGGAGCGACCACT
CTCCCTGGATACGGTGGCTTTGCTGAAGGAGGCTCTTGTGGCTGGCAGCTATGCGGCTGC
TGTTTCGGGACTACCAGAGGATGCCAAGCCACTGATGCGACTACTGCCATTGACTACTGC
CTTAGCCTCGCGACCAATCCTCGCAAAGGTGGCCGATATAAGCGTGGGTCTCTCTCATGA
AACCCTGCACAATACCAGCATGATGCTGCTCTCGCCACAGCAGCGCTTTCACCTTATCG
CACGGACATCTGGACTCGCTTATGGGACCTTCTCAACGAGTCAGCCAAAGCAGGAGCGC
TAGATTCACTGCTGAGCAGGTGACGGAGAAGTTGATCTTTAGTTTGGCTGCTACCAGCC
GGAGGCTCTGTCCAGATGCACCACGCCACTTTCGCCAGACACGGGCACCGGTGGATTTGG
TGACTATAGTAGCGGAAGTGCTTTTCCATTTCAGCAACGAAGTGCTGCCCTTTATCGAACT
GGAGGGATGCACAGCCAGCAGCAGGAGCAGTCAATTTCTGTGTATCTGCGCGAGCTGAG
CGTTACCTGGTGAAGCACACGTCAAAGCCCAACACTGGCTTCCGTTGGCTGAAGGAGAC
CTTTTTCGAGCGCTCCAGGCTCCAGCTCATGTGCATGCGGTGGCTCGCAGTTGCTTTC
CGCGCAGCTTGAATCTCGCGGGCTCTTGTCTCTGCTTGTGCTGCTGCGAGGCTTAGA
TGCGCGCATGGAGACCTCGAGGGGTTTTAGTTGATTGACCAATGGCTGCCAATCAACA
GCACTCTCTGTTTCTAATCCTCGAGCGCTATTGCTGGCTGTGGAATCAATTGCGTTTCC
CCTGCCCGAGGGTTTCTCCTCGTTCTTCACTACTTGGCTATCGTGCGCTGGGCTATGA
TATGTTTCTGCAATATGTGGAATCATGTGTTTGAAGTGAAGTGGATGTGATGAAGGC
CATGTTTTCGATATTGAAGATTCTCCACTAGGCATTGAGCGGAAGCTATCACTTTTGT
TGCTCTGCCCAAGCAGCGTCTCAAAGGTTACTCAAATGCTGGCAGCATCCGGACAGCCT
TATGATCCGCGACGCGAGCATGCGGCCAACATTCTGTGCGGTCAGCAGCAGGAGGTGTT
GCACCAGCAGCGACCCACGGCCTGCGTGAATCAATCGCGAAATAATGGCCGGAGCGATCT
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TCTAAACGAATTGGACTACAATCTGCTTATTGAACTACTCTAAGCTCCATCGATCAGCT
GAAACTGGAGGCATGAATTTAATGTTAAGAGTAACTAATGAAGTATTGTGTCAAATTTATC
AAGTACTTAGCCAAGGCCAGTTTGCAAATATCCAAAGATTTGATTTGTCAAATGTATTA
GTTAAGATTCTTCTCGTGCAGCTTTGATTTTGTAGGGTTCTTCTGTGTGCTTTAGTA
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Fig. 3A

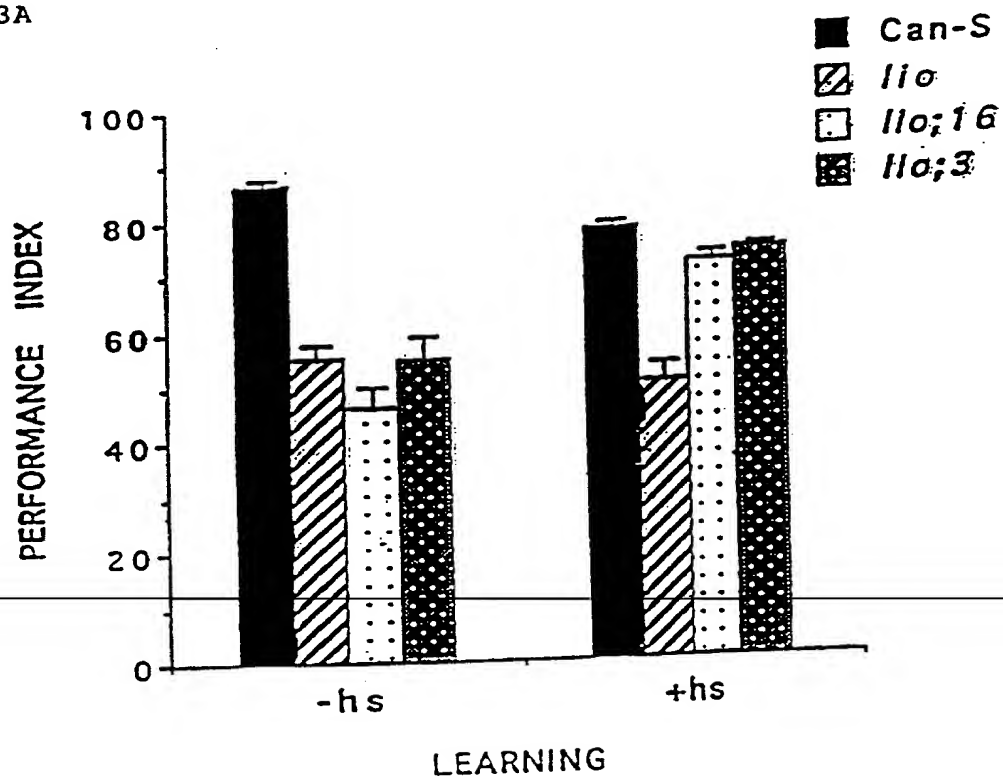
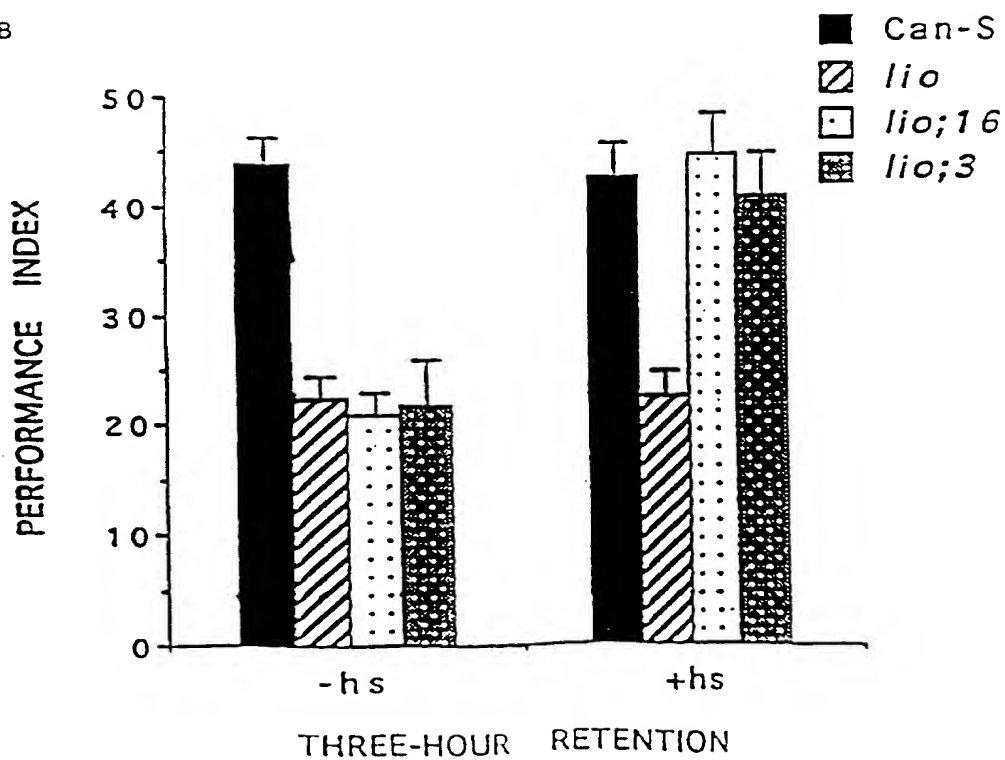


Fig. 3B



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Fig. 4A

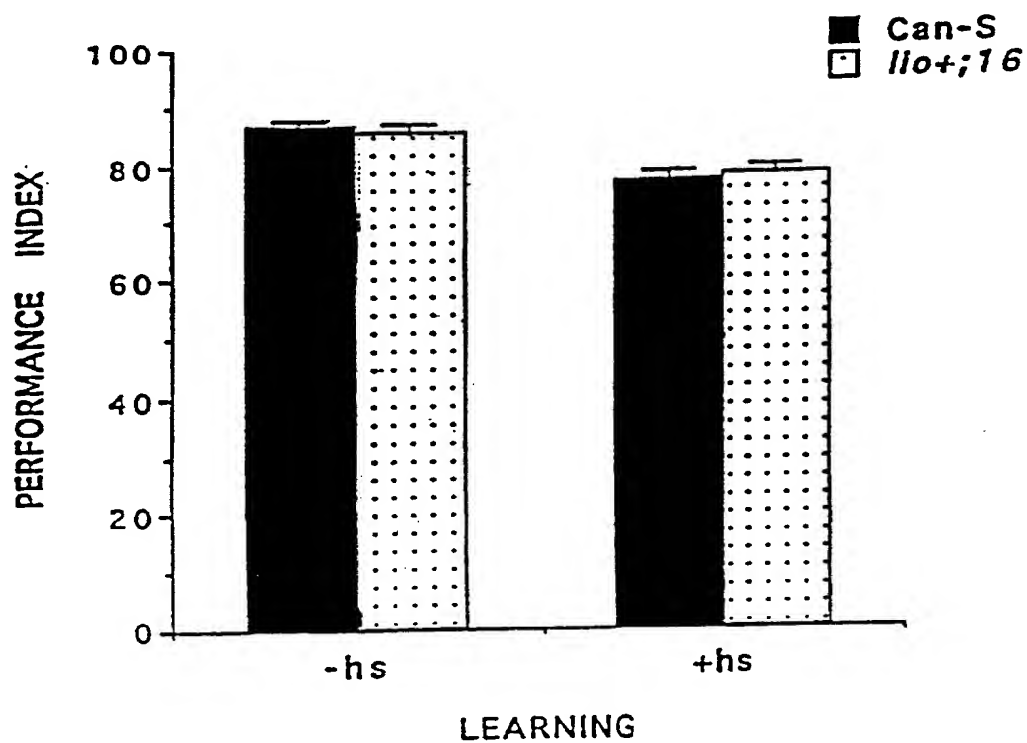


Fig. 4B

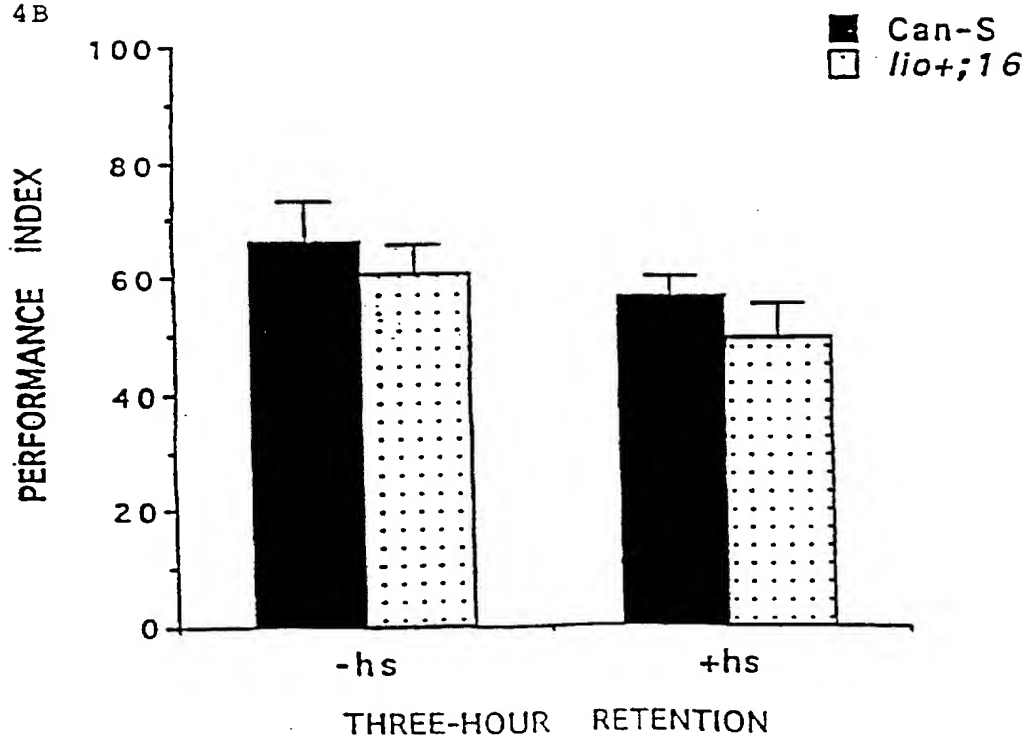


Fig. 5A

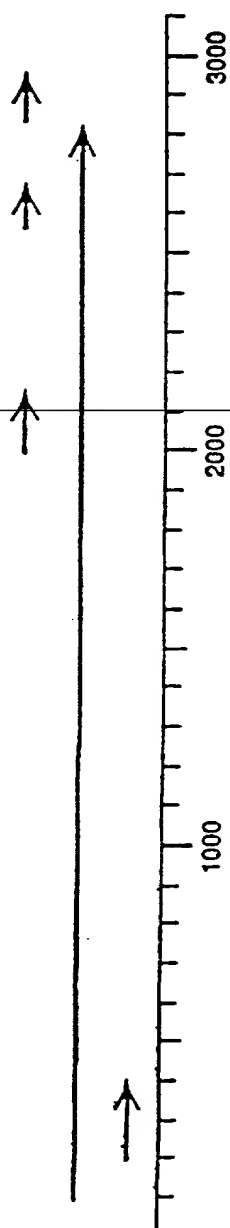
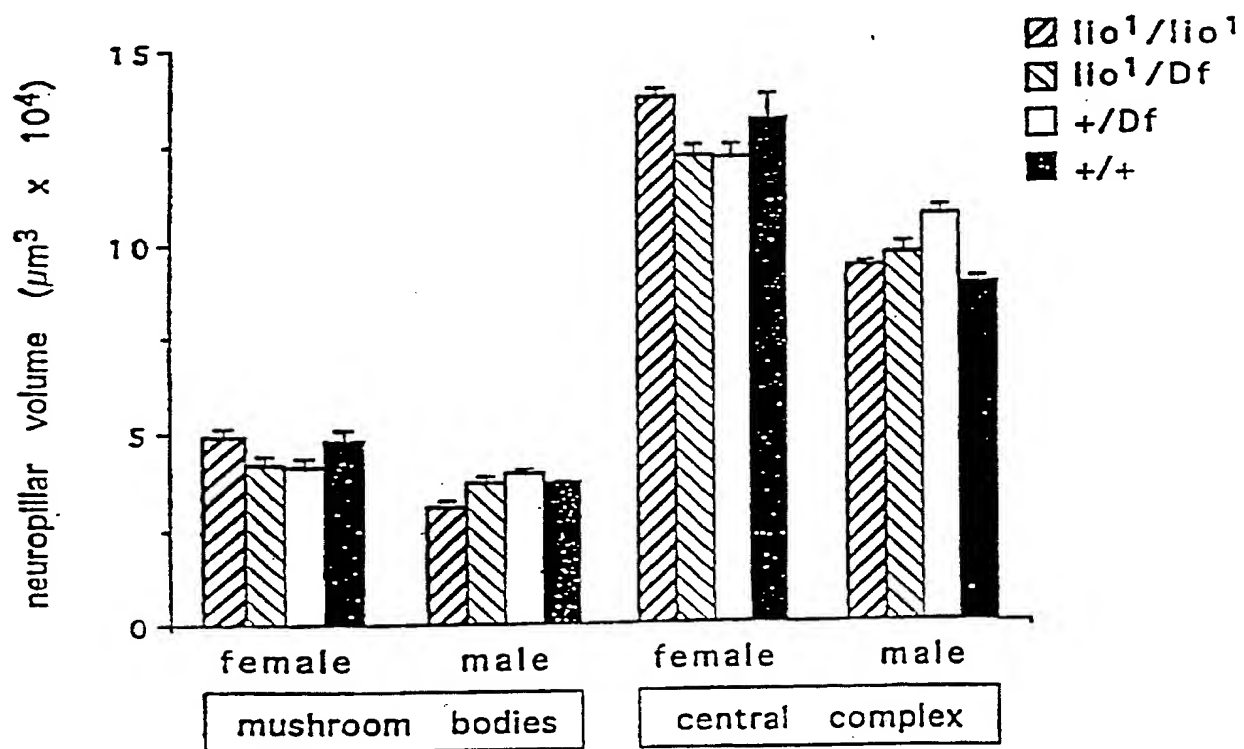


Fig. 5B

HLRQENLAANFCGLLASQGYKEKANEWRI LGQEQDGSLLTSHIFEYADEDQDKETCIGHFHATKKQLRLWLTDNCHREIVQATINSS
 VTLLSFVEKTEGKLYQAFVVEVSSEGGTATPLNSEPSNRQNTQFLWRVESATRTWCQDKLLVLTHEESIKQYSCVVKQSSTTCST
 GCGEGSAWRDLTSLITYETLARNFSWAQWDPCEQALYYIHLKPKAKSLSLDDEEEAGEQTPTLSPTLSAFQFNEKQPTETVLNIP
 LNLPKLPNGSKEEPSYDDDAVPLRVHDSLSNLIILADTSGHFFVCHYLYQPMQSEQRDVHFAYSVTLHHGCVVHCVMFGVPWQK
 ARLLRPTFALHGQHLLVSSAFFVHLLDVGLQHEPNCHIVCAHNRSPTITQLVPLRKWGALAYDAATLDLVSLSVPKSHLIEAFRN
 DSSLDNRISIIHYFLFDSNDMDVLAELLNNILERPISLDTVALKEALVAGSYAAAVRGLPEDAKPLMRLLPLTTALASRPILAKVA
 DISVGLSHETLHNTSNMULLSPQQRSLSPYRTDIWTRLWDLNESAQEQPFSAEQVTEKLIIFSLACYQPEALSRCTTPLSPDTGTGG
 FGYSYSGSAFPFSNEVLPIELEGCTASKQEHVTSVYLRSLSVHLVKHTSKENTGFRWLKETFFERSQAPAHVHAVASQFVSAQLEL
 SRALCSLVCRAAGLDARMETSRGFQIDQMAANOQHSLFLILERYCLAVESIAFPLPEGFSSFFTYLGYRALGYDMFLQYVENHVFE
 LQVDVHKAIIVFDIEDSPGIERKLSLLSALPKQRAQRLLKCWQHPDLSLMIRGREHAANILSCQQQEVHLHQORPTACVNOGRNGRSD
 LTAEALSPLDSFSLDLTAKASLNELDYNLLIETTLLSSIDQLKLEAZ

Fig. 6



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/15853

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/435 C07K16/18 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEURON (1995), 15(4), 829-42 CODEN: NERNET;ISSN: 0896-6273,	1-11
A	1 October 1995, XP000616509 BOLWIG, GERT M. ET AL: "Molecular cloning of linotte in Drosophila: a novel gene that functions in adults during associative learning" see the whole document --- FEBS LETT. (1995), 370(3), 250-4 CODEN: FEBLAL;ISSN: 0014-5793, 1995, XP002024037 DURA, JEAN-MAURICE ET AL: "The Drosophila learning and memory gene linotte encodes a putative receptor tyrosine kinase homologous to the human RYK gene product" see the whole document --- -/-	1-11



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Date of the actual completion of the international search

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12.02.97

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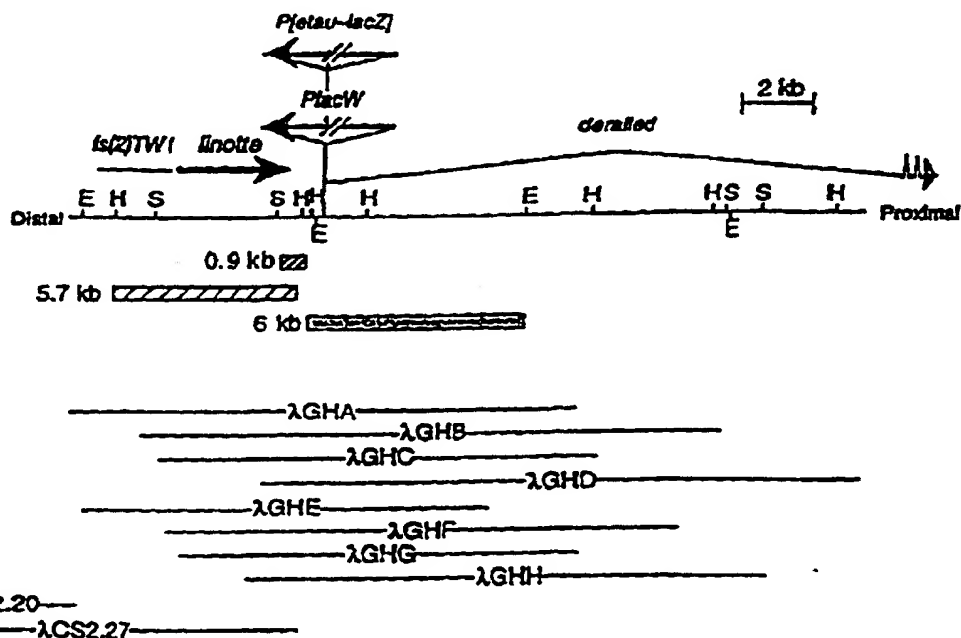
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. NEUROGENETICS, vol. 9, no. 1, 1993, ELSEVIER, AMSTERDAM, NL, pages 1-14, XP000616504 J.M. DURA ET AL.: "Identification of Linotte, a new gene affecting learning and memory in Drosophila melanogaster" cited in the application see the whole document ---	1-11
A	GENETICS (1992), 131(3), 655-72 CODEN: GENTAE;ISSN: 0016-6731, 1992, XP000616518 BOYNTON, SUSAN ET AL: "Latheo, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis" cited in the application see the whole document ---	1-11
A	TRENDS IN NEUROSCIENCE, vol. 18, no. 5, May 1995, ELSEVIER, CAMBRIDGE, UK, pages 212-218, XP000616503 J. DEZAZZO AND T. TULLY: "Dissection of memory formation: from behavioral pharmacology to molecular genetics" cited in the application see the whole document -----	1-11



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agents: GRANAHAAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).			

(54) Title: ASSOCIATIVE LEARNING AND THE LINOTTE GENE



(57) Abstract

The invention pertains to a novel gene present in cytological region 37D of the second chromosome which functions in associative learning and/or memory. Disruption of the gene, such as by P element transposon tagged insertion, results in decreased associative learning and/or memory. The invention also pertains to a novel protein encoded by the gene, antibodies which bind the encoded protein, and homologs of the novel gene which function in associative learning and hybridize to the DNA sequence of the novel gene.

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